A Protein Having DNase Activity

The present invention relates to a protein having DNase activity, a DNA encoding the same and a process for the preparation thereof. In addition, the invention concerns the use of the DNA and the protein as well as antibodies directed against the protein.

It is known that many cells suffer from programmed cell death. This cell death is referred to as apoptosis. It is found e.g. in the case of organogenesis and metamorphosis, tissue atrophy and tumor regression. Apoptosis is accompanied by a condensation of cytoplasm, a loss of plasma membrane villi, a segmentation of the nucleus and particularly an extensive degradation of chromosomal DNA. The latter manifests itself in that a "ladder" of DNA fragments, particularly having a size of over 600 kb, 50-300 kb and 50 kb, is present in apoptotic cells. It has not been known by now which mechanisms are responsible for the degradation of chromosomal DNA. However, this would be necessary to better understand apoptosis so as to be optionally able to take measures for or against it.

Thus, it is the object of the present invention to provide a product by which the degradation of chromosomal DNA in apoptotic cells can be investigated.

According to the invention this is achieved by providing the subject matters as defined in the claims.

Therefore, the subject matter of the present invention relates to a protein having DNase activity, which comprises the amino acid sequence of fig. 1 or a functional derivative or fragment thereof.

The expression "DNase activity" refers to the fact that the protein can cut single-stranded and/or double-stranded DNA.

The expression "functional derivative or fragment" comprises any derivative or fragment of the amino acid

sequence of fig. 1, which has DNase activity. The amino acid sequence of fig. 1 may also include additions, substitutions and/or deletions of one or more amino acids, which also applies to the functional derivatives or fragments thereof.

A further subject matter of the present invention relates to a nucleic acid coding for an above protein. This may be an RNA or a DNA. The latter may be e.g. a genomic DNA or a cDNA. Preferred is a DNA which comprises the following:

(a) the DNA of fig. 1 or a portion thereof,
(b) a DNA hybridizing with the DNA of (a), or
(c) a DNA related to the DNA of (a) or (b) via the degenerated genetic code.

The expression "hybridizing DNA" refers to a DNA hybridizing with a DNA of (a) under normal conditions, particularly at 20°C below the melting point of the DNA.

The DNA of fig. 1 was deposited with DSM (*Deutsche Sammlung von Mikroorganismen und Zellkulturen* [Germany-type collection of microorganisms and cell cultures]) as JFC4 under DSM 9993 on May 23, 1995.

A DNA according to the invention is described below in the form of a cDNA. It is exemplary for every DNA falling under the present invention.

For the production of a cDNA according to the invention it is favorable to use a cosmid library, e.g. q1Z (cf. Dietrich, A. et al., Nucleic Acids Res. 19, (1991), 2567-2572), as a basis, clones of which comprise the region Xq27.3-Yqter of the human genome. Such clones are fixed on a filter membrane and hybridized with labeled cDNA pools obtained from mRNA of pig tissues, e.g. brain, muscle, liver, by reverse transcription (cf. Coy, J.F. et al., Mammalian Genome 5, (1994) 131-137). Those clones having a hybridization signal with the cDNA pools are used for

screening a human cDNA library, e.g. of fetal cerebral tissue. For this purpose, particularly the cDNA library - Zap, Stratagene company, catalog No. 936206 is suitable. A cDNA according to the invention is obtained.

A cDNA according to the invention can be present in a vector and expression vector, respectively. A person skilled in the art is familiar with examples thereof. In the case of an expression vector for E. coli these are e.g. pGEMEX, pUC derivatives, pGEX-2T, pET3b and pQE-8, the latter being preferred. For the expression in yeast e.g. pY100 and Ycpad1 have to be mentioned, while for the expression in animal cells e.g. pKCR, pEFBOS, cDM8 and pCEV4 have to be indicated. The baculovirus expression vector pAcSGHisNT-A is especially suitable for the expression in insect cells.

The person skilled in the art knows suitable cells to express a cDNA according to the invention, which is present in an expression vector. Examples of such cells comprise the E. coli strains HB101, DH1, x1776, JM101, JM109, BL21, and SG 13009, the latter being preferred, the yeast strain saccharomyces cerevisiae and the animal cells L, 3T3, FM3A, CHO, COS, Vero and HeLa as well as the insect cells sf9.

The person skilled in the art knows in which way a cDNA according to the invention has to be inserted in an expression vector. He is also familiar with the fact that this DNA can be inserted in combination with a DNA coding for another protein and peptide, respectively, so that the cDNA according to the invention can be expressed in the form of a fusion protein.

In addition, the person skilled in the art knows conditions of cultivating transformed cells and transfected cells, respectively. He is also familiar with processes serving for isolating and purifying the protein expressed by the cDNA according to the invention. Thus, such a protein which

may also be a fusion protein also belongs to the subject matter of the present invention.

A further subject matter of the present invention relates to an antibody directed against an above protein and fusion protein, respectively. Such an antibody may be prepared by common methods. It may be polyclonal and monoclonal, respectively. For its production it is favorable to immunize animals - particularly rabbits or chickens for a polyclonal antibody and mice for a monoclonal antibody - with an above (fusion) protein or fragments thereof. Further "boosters" of the animals may take place with the same (fusion) protein or fragments thereof. The polyclonal antibody may then be obtained from the animal serum and egg yolk, respectively. For the monoclonal antibody, spleen cells of the animals are fused with myeloma cells.

A preferred antibody of the present invention, namely the monoclonal antibody 11/78/1, was deposited with DSM under DSM ACC 2211 on April 26, 1995.

The present invention enables to investigate the degradation of chromosomal DNA in apoptotic cells. This investigation can be carried out with a person's isolated body fluids. A DNase responsible for the above degradation can be detected by the antibody according to the invention. Furthermore, an autoantibody directed against this DNase can be detected by a protein according to the invention. Both detections may be made by common methods, particularly a Western blot, an ELISA, an immunoprecipitation or by immunofluorescence. In addition, the expression of the gene coding for the above DNase can be detected by a nucleic acid according to the invention, particularly a DNA and primers derived therefrom. This detection may be made as usual, particularly in a Southern blot.

Moreover, the present invention is suitable to take measures for or against apoptosis. These measures comprise the administration of a product according to the invention

to a person. An above DNase can be inhibited by an antibody according to the invention so as to prevent the degradation of chromosomal DNA. On the other hand, this degradation can be promoted by a protein according to the invention, particularly after linkage to a protein which is not considered foreign by the body, e.g. transferrin or BSA, which would be especially suitable for the treatment of tumor cells. The same can be achieved correspondingly with a nucleic acid according to the invention, particularly a DNA which is controlled by a promoter inducible in certain tissues, e.g. tumors, and results in the provision of a protein according to the invention in these tissues after the expression thereof. Moreover, a nucleic acid according to the invention, particularly a DNA, can also be used for inhibiting an above DNase. For this purpose, the nucleic acid is used e.g. as a basis for the preparation of anti-sense oligonucleotides for the expression inhibition of the gene coding for the above DNase.

Thus, the present invention represents a major contribution to the diagnostic and therapeutic detection or registration of apoptosis.

Brief description of the drawing:

Fig. 1 shows the base sequence and the derived amino acid sequence of a protein according to the invention which has DNase activity. (SEQ ID NO: 1)

The present invention is explained by the below examples.

Example 1: Preparation and purification of a protein according to the invention

The DNA of fig. 1 was used as template for the preparation of a protein according to the invention. A PCR method was carried out. The primer pair used was: 5'-CAGGGATCCGATGACGATGACAAAATGCACTACCCAACTGCAC-3' and 5'-

GGGGGATCCTCAGGCAGCAGGGCACAG-3' (SEQ ID Nos 1 and 2, respectively). The PCR supported batch approach and the PCR conditions were as follows:

PCR batch

template DNA (fig. 1)	: 1 µl = 1 ng
Pfu polymerase 10x buffer	: 10 µl = 1 x
DMSO	: 10 µl = 10 %
dNTPs	: 1 µl = 200 µM each
oligonucleotides, 1.5 µl each	: 3 µl = 150 ng each
H_2O bidistilled	: ad 99 µl

PCR conditions

- 92°C - 5 min
- addition of 1 µl Pfu polymerase (Stratagene company) = 2.5 units
- addition of paraffin

PCR

92°C	1 min	
58°C	1 min	1 cycle
72°C	10 min	
92°C	1 min	
58°C	1 min	39 cycles
72°C	2 min	
72°C	10 min	1 cycle

The amplified DNA was cleaved by BamHI and inserted in the only BamHI site of the expression vector pQE-8 (Qiagen company). The expression plasmid pQ/DNaseX was obtained. It codes for a fusion protein comprising 6 histidine residues (N terminus partner) and the protein of fig. 1 according to the invention (C terminus partner). pQ/DNaseX was used for the transformation of E. coli SG 13009 (cf. Gottesman, S. et al., J. Bacteriol. 148, (1981), 265-273). The bacteria were cultivated in an LB medium with 100 µg/ml ampicillin and 25 µg/ml kanamycin and induced with 60 µM isopropyl-ß-D-thiogalactopyranoside (IPTG) for 4 h. Lysis of the bacteria was achieved by the addition of 6 M guanidine hydrochloride. Thereafter, chromatography (Ni-NTA resin)

was carried out with the lysate in the presence of 8 M urea in accordance with the instructions of the manufacturer (Qiagen company) of the chromatography material. The bound fusion protein was eluted in a buffer having pH 3.5. After its neutralization, the fusion protein was subjected to an 18 % SDS polyacrylamide gel electrophoresis and dyed with coomassie blue (cf. Thomas, J.O. and Kornberg, R.D., J. Mol. Biol. 149 (1975), 709-733).

It showed that a highly pure form of a (fusion) protein according to the invention can be prepared.

Example 2: Preparation of an antibody according to the invention

A fusion protein of Example 1 according to the invention was subjected to an 18 % SDS-polyacrylamide gel electrophoresis. After dyeing the gel with 4 M sodium acetate, a 35 kD band was cut out of the gel and incubated in phosphate-buffered common salt solution. Gel pieces were sedimented before the protein concentration of the supernatant was determined by an SDS polyacrylamide gel electrophoresis which was followed by coomassie blue dyeing. Animals were immunized with the gel-purified fusion protein as follows:

Immunization protocol for polyclonal antibodies in rabbits

35 μg of gel-purified fusion protein in 0.7 ml PBS and 0.7 ml complete Freund's adjuvant and incomplete Freund's adjuvant, respectively, were used per immunization.

Day 0: 1st immunization (complete Freund's adjuvant)
day 14: 2nd immunization (incomplete Freund's adjuvant; icFA)
day 28: 3rd immunization (icFA)
day 56: 4th immunization (icFA)
day 80: bleeding to death.

The rabbit serum was tested in an immunoblot. For this purpose, a fusion protein of Example 1 according to the invention was subjected to an SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter (cf. Khyse-Andersen, J., J. Biochem. Biophys. Meth. 10 (1984), 203-209). The Western blot analysis was carried out as described in Bock, C.-T. et al., Virus Genes 8, (1994), 215-229. For this purpose, the nitrocellulose filter was incubated with a first antibody at 37°C for one hour. This antibody was the serum of the rabbit (1:10000 in PBS). After several wash steps with PBS, the nitrocellulose filter was incubated with a second antibody. This antibody was a monoclonal goat anti-rabbit-IgG antibody linked with alkaline phosphatase (Dianova company) (1:5000) in PBS. Incubation at 37°C for 30 minutes was followed by several wash steps with PBS and then by the alkaline phosphatase detection reaction with developer solution (36 μM 5'-bromo-4-chloro-3-indolylphosphate, 400 μM nitroblue tetrazolium, 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) at room temperature until bands were visible.

It showed that polyclonal antibodies according to the invention can be produced.

Immunization protocol for polyclonal antibodies in chickens

40 μg of gel-purified fusion protein in 0.8 ml PBS and 0.8 ml complete Freund's adjuvant and incomplete Freund's adjuvant, respectively, were used per immunization.

Day 0: 1st immunization (complete Freund's adjuvant)
day 28: 2nd immunization (incomplete Freund's adjuvant; icFA)
day 50: 3rd immunization (icFA)

Antibodies were extracted from egg yolk and tested in a Western blot. Polyclonal antibodies according to the invention were detected.

Immunization protocol for monoclonal mouse antibodies

12 µg of gel-purified fusion protein in 0.25 ml PBS and 0.25 ml complete Freund's adjuvant and incomplete Freund's adjuvant, respectively, were used per immunization. In the 4th immunization the fusion protein was dissolved in 0.5 ml (without adjuvant).

Day 0: 1st immunization (complete Freund's adjuvant)
day 28: 2nd immunization (incomplete Freund's adjuvant; icFA)
day 56: 3rd immunization (icFA)
day 84: 4th immunization (PBS)
day 87: fusion.

Supernatants of hybridomas were tested in a Western blot. Monoclonal antibodies according to the invention were detected. One of them, 11/78/1, was deposited with DSM under DSM ACC 2211 on April 26, 1995.

Example 3. Detection of the DNase activity of a protein according to the invention

A DNase activity test was made according to the method by Rosenthal, A.L. & Lacks, S.A., Anal. Biochem. 80, (1977), 76-90, with modifications. For this purpose, an 18 % SDS polyacrylamide gel was produced which contained 2 mM EDTA and denatured salmon testis DNA or yeast RNA up to a final concentration of 10 µg/ml in the separation and collection gel. Samples were denatured by boiling them in Laemmli sample buffer, which contained 5 % of 2-mercaptoethanol, for 4 min. A protein according to the invention (from Example 1) and bovine DNase 1 (control) were used as samples. A 10 kd ladder (Gibco BRL company) was used as protein marker, which was separated in the same gel, cut out after electrophoresis and dyed with coomassie blue. For removing the SDS after the electrophoresis, the gel containing the samples was washed with 100 ml 40 mM Tris-HCl, pH 7.6, for 4 x 30 min and incubated in 40 mM Tris-

HCl, pH 7.6, with 0.02 % sodium azide and 2 mM $MgCl_2$, 2 mM $CaCl_2$ and with 2 mM $MgCl_2$, 2 mM $ZnCl_2$, respectively, at room temperature overnight. For detecting the enzymatic activity, the buffer was changed and ethidium bromide was added up to a final concentration of 2 μg/ml. The gel was investigated periodically on a long-wave U.V. light and photographed.

It showed that a protein according to the invention has DNase activity.